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COMPARATIVE ASSESSMENT OF VARIOUS ANTIBIOTICS FOR CONTROLLING BACTERIAL BLIGHT IN *EUCALYPTUS CAMALDULENSIS*

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ABSTRACT

Eucalyptus camaldulensis is a significant tree species found in tropical, subtropical, and temperate regions worldwide. It belongs to the family Myrtaceae and is native to Australia. While numerous diseases affect Eucalyptus production, bacterial blight stands out as one of the most serious diseases, causing severe damage during the nursery stage. There is a strong link between environmental factors and disease incidence. After collecting diseased samples from the field, various experiments were conducted in the research area of the Department of Forestry and Range Management at the University of Agriculture, Faisalabad. The primary objective of this study was to evaluate different concentrations of antibiotics for managing bacterial blight disease through antibiotic treatments. Following isolation, identification, and pathogenicity tests, antibiotics such as Amikacin Sulphate, Cefuroxime, Ceftizoxime Sodium, Co-amoxiclav, Ceftriaxone, Cephradine, Ampicillin Sodium, Ampicillin/Cloxacillin, and Ceftazidime, along with a control treatment, were assessed at concentrations of 300 ppm, 500 ppm, and 700 ppm under *in vitro*, greenhouse, and *in vivo* conditions. Among these, Ceftriaxone exhibited the best performance, forming the maximum inhibition zone (12.657 mm) at concentrations of 300 ppm, 500 ppm, and 700 ppm in the *in vivo* experiment, compared to the aforementioned treatments. Meanwhile, the lowest disease incidence (%) was recorded in the greenhouse (15.185%) and *in vivo* (18.519%) experiments at concentrations of 300 ppm, 500 ppm, and 700 ppm, using the combination of Cephradine + Ceftriaxone. The study underscores the effectiveness of Ceftriaxone and the Cephradine + Ceftriaxone combination as promising antibiotic treatments for managing this disease.

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INTRODUCTION

Eucalyptus camaldulensis is an essential and versatile tree species, encompassing more than 900 varieties and originating from Australia (Dhakad et al., 2018). Currently, there are approximately 20 million hectares of plantations spread across over 90 countries worldwide. These plantations serve as a source of industrial raw materials like paper, pulp, sawn timber, charcoal, wood panels, as well as woodlots dedicated to producing charcoal and firewood for domestic use (FAO,

2018). Eucalyptus thrives in diverse environmental conditions, exhibiting a remarkable ability to endure drought. Numerous species within this genus are recognized for their capacity to withstand water scarcity and adapt to conditions of extremely low water potential (Merchant et al., 2007). Furthermore, Eucalyptus plantations play a significant role in enhancing the physico-chemical attributes of problematic soils. This is attributed to the exceptional capacity of the tree for nutrient uptake, ultimately leading to soil structure improvement (Mishra et al., 2003). Eucalyptus is renowned for the therapeutic attributes of the essential oils found in its leaves. These essential oils have applications in treating wounds and fungal infections. Extracts from Eucalyptus leaves are commonly utilized in the food, perfumery, and pharmaceutical industries. Utilizing Eucalyptus essential oils as a pesticide, offers an environmentally friendly alternative to synthetic pesticides (Batish et al., 2008).

Numerous diseases are caused by bacteria, viruses, and fungi that attack Eucalyptus plantations. Bacterial and fungal pathogens that lead to significant destruction in Eucalyptus plantations include *Puccinia psidii*, which causes Eucalyptus rust (Glen et al., 2007). In the year 2009, *Teratosphaeria nubilosa* was identified as the causal organism responsible for the severe leaf blotch disease in Eucalyptus (Perez et al., 2009). *T. nubilosa* is a significant leaf pathogen of Eucalyptus in Australia (Hunter et al., 2009). The pathogen responsible for bacterial blight in Eucalyptus was identified as *Xanthomonas campestris* pv. *eucalypti* (Truman, 1974). Moreover, in 2002, *Pantoea ananatis* was also identified as a causal agent of bacterial blight (Coutinho et al., 2002). Among these pathogens, *X. axonopodis* pv. *eucalyptorum* stands out as the most damaging pathogen, causing bacterial blight disease in Eucalyptus plantations and resulting in severe economic losses (Mooter et al., 1987). In nurseries and newly established plantations, dieback and bacterial blight of Eucalyptus species have become increasingly problematic over the last decade in various regions around the world (Coutinho et al., 2011). This disease profoundly affects plant growth (Procopio et al., 2009). Bacterial blight of Eucalyptus has led to substantial economic losses for commercial nurseries. Similarly, infections in young trees have resulted in a high mortality rate during extreme field outbreaks (Ferreira et al., 2008).

The typical indications of bacterial blight disease include tip dieback and spots on immature leaves. The symptoms on the leaves begin as water-soaked lesions. In more severe cases, these lesions expand in size due to excess moisture, resulting in damage to the leaves and eventual defoliation. Initially, the leaf lesions appear pale yellow and later turn brown. The bacterial pathogen tends to spread from the petiole to adjacent leaf tissues, often clustering around the major leaf veins. This progression leads to the death of petioles and premature leaf abscission. In the advanced stages of bacterial blight-infected trees, scorched symptoms and repeated infections stunt tree growth. The trees adopt a bushier appearance due to the proliferation of newly growing tips and adventitious shoots. Sensitive tree species, hybrids, and clones display both dieback and blight signs. However, more tolerant species only exhibit leaf spot symptoms (Coutinho et al., 2002). Two distinct bacterial genera, *Xanthomonas* and *Pantoea*, have been isolated from Eucalyptus trees displaying bacterial blight symptoms (Swart, 2010). Recent studies have identified several *Xanthomonas* species responsible for causing bacterial blight in Eucalyptus. For instance, in Australia, *X. dyei* pv. *eucalypti* and *X. campestris* pv. *eucalypti* have been identified, while *X. axonopodis* pv. *eucalyptorum* and *X. vasicola* were found in Brazil and Uruguay (Bophela et al., 2019). In the current study focused on managing bacterial blight in *Eucalyptus camaldulensis*, antibiotics at varying concentrations were evaluated.

MATERIAL AND METHODS

Collection, isolation, purification, identification of *X. axonopodis* pv. *eucalyptorum*

Diseased samples exhibiting bacterial blight symptoms were collected from the Shorkot Irrigated Forest Plantation in Pakistan. These samples were subsequently transported to the Bacteriology Lab of the Plant Pathology department at UAF Pakistan for the purpose of isolating *Xanthomonas axonopodis* pv. *eucalyptorum*.

To prepare the nutrient agar media (NA), 20 g of nutrient agar was accurately weighed and then dissolved in 1000 ml of distilled water within a media bottle. The mixture was thoroughly shaken to ensure proper dissolution. Once the nutrient agar was fully dissolved, it underwent sterilization in an autoclave at a temperature of 121°C. In preparation for sterilization, petri plates were washed, dried, and subsequently wrapped in newspaper. These plates were then subjected to

autoclaving at the same temperature. The sterilized NA was poured into these prepared petri plates.

Diseased tissue from the samples, along with some healthy tissue, was carefully excised into small pieces of approximately 1 cm. These tissue segments were then subjected to surface sterilization using a 4% sodium hypochlorite (NaOCl) solution for a duration of 30 seconds. Once the media had properly set, the tissue samples were placed onto the petri plates containing the NA. These plates were wrapped and placed in an incubator set to 28°C for a period of 24-48 hours. Following this incubation, the growth of the pathogen was observed and assessed within the specified time frame. The isolated pathogen was subsequently subjected to purification procedures. Identification of the pathogen was accomplished through observation of morphological characteristics.

To assess the pathogenicity of *X. axonopodis* pv. *eucalyptorum*, *E. camaldulensis* plants were cultivated within the experimental area of the Department of Forestry and Range Management at UAF. A bacterial inoculum suspension with a concentration of 1×10^5 colony-forming units per milliliter (cfu/ml) was evenly sprayed onto the plants. Disease symptoms manifested on the leaves and shoots of the plants approximately 2 months after inoculation. The initial symptoms consisted of water-soaked lesions, which later progressed to necrotic areas as the disease advanced (Coutinho et al., 2015). Subsequent to symptom development, the pathogen was isolated and then re-isolated in accordance with the requirements of Koch's postulates for disease causation.

***In vitro* assessment of antibiotics against *X. axonopodis* pv. *eucalyptorum* through inhibition zone technique**

Nine antibiotics, namely Ceftriaxone, Cephradine, Ampicillin/Cloxacillin, Ampicillin sodium, Cefuroxime, Ceftizoxime Sodium, Amikacin Sulphate, Co-amoxiclav, and Ceftazidime, were assessed against *X. axonopodis* pv. *eucalyptorum*. Stock solutions of antibiotics were prepared, and three concentrations of each antibiotic were utilized. To prepare the stock solutions, 1 g of antibiotic was added to 100 ml of distilled water. For subsequent preparation of concentrations of 300 ppm, 500 ppm, and 700 ppm, 3 ml, 5 ml, and 7 ml of the respective stock antibiotic solutions were added to 1000 ml of distilled water.

Filter paper pieces, each measuring 1 cm, were cut and then autoclaved. NA was poured into sterilized petri plates, and a bacterial suspension of 1×10^5 cfu/ml was streaked on NA using a cotton swab. Filter papers were immersed in the required antibiotic concentration for 30 seconds and then positioned in the center of the streaked petri plates, with a control treatment included for each concentration. The petri plates were subsequently placed in an incubator set at 28°C. The inhibition zones produced by each antibiotic were measured using a Digital Vernier caliper after 12, 24, and 36 hours, and the measurements were recorded accurately. The experimental design followed a Completely Randomized Design with three replications.

Assessment of antibiotics for the management of bacterial blight of Eucalyptus under greenhouse conditions

To assess the efficacy of antibiotics in disease control, two antibiotics, namely Cephradine and Ceftriaxone, which exhibited the most substantial inhibition zones in the *in vitro* assessment, were chosen for the greenhouse experiment. Ceftriaxone, Cephradine, and a combination of both (Cephradine + Ceftriaxone) were employed, alongside a control treatment, to combat the bacterial disease. Initial concentrations of each treatment were set at 300 ppm, 500 ppm, and 700 ppm. Sandy loam soil was used to fill the pots. The designated concentrations of each antibiotic were then applied through spraying onto the infected Eucalyptus plants. Data regarding disease incidence was collected at 10, 20, and 30-day intervals to assess the progression of the disease. The experimental design followed a Randomized Complete Block Design with three replications.

***In vivo* evaluation of antibiotics for the control of bacterial blight of Eucalyptus**

To evaluate the effectiveness of antibiotics against bacterial blight in Eucalyptus through *in vitro* methods, previously established diseased Eucalyptus plants that had been utilized for pathogenicity tests were employed. The field experiment comprised three treatments: Cephradine, Ceftriaxone, and a combination of both (Cephradine + Ceftriaxone), along with a control treatment. Three concentrations of each antibiotic were prepared (300 ppm, 500 ppm, and 700 ppm). These concentrations were administered through spraying onto the affected plants, and data were recorded at various intervals (10, 20, and 30 days). The experimental layout followed a completely randomized block design, with three replications.

Data analysis

All laboratory and greenhouse experiments were carried out using a Completely Randomized Design, while the field experiment was conducted employing a Randomized Complete Block Design, utilizing Minitab version 18.1. To differentiate means among various treatments, the Least Significant Difference (LSD) at a 5% significance level was utilized.

RESULTS

***In vitro* assessment of antibiotics against *X. axonopodis* pv. *eucalyptorum* through inhibition zone technique**

Among all ten treatments, Ceftriaxone exhibited the maximum inhibition zone (13 mm), followed by Cephradine, Ampicillin/Cloxacillin, Ampicillin sodium, Cefuroxime, Ceftizoxime Sodium, Amikacin Sulphate, Co-amoxiclav, and Ceftazidime when compared to the control treatment (Figure 1). Regarding the interaction between concentration and treatments, Ceftriaxone displayed the largest inhibition zone (11 mm, 13 mm, and 14 mm) at concentrations of 300 ppm, 500 ppm, and 700 ppm, respectively. This was followed by Cephradine, Ampicillin/Cloxacillin, Ampicillin Sodium, Cefuroxime, Ceftizoxime Sodium, Amikacin Sulphate, Co-amoxiclav, and Ceftazidime at the corresponding concentrations, compared to the control treatment (Figure 2a).

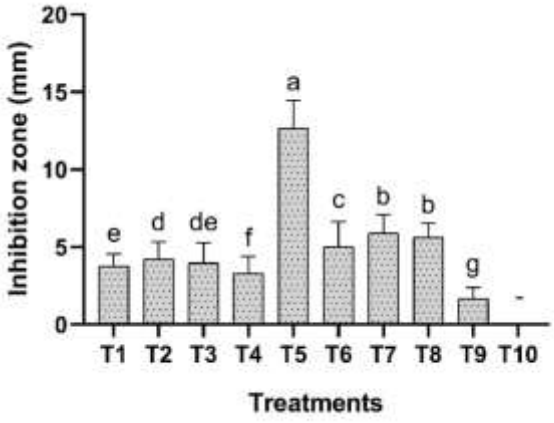

Figure 1: *In vitro* evaluation of antibiotics for inhibition zone against *X. axonopodis* pv. *eucalyptorum.*

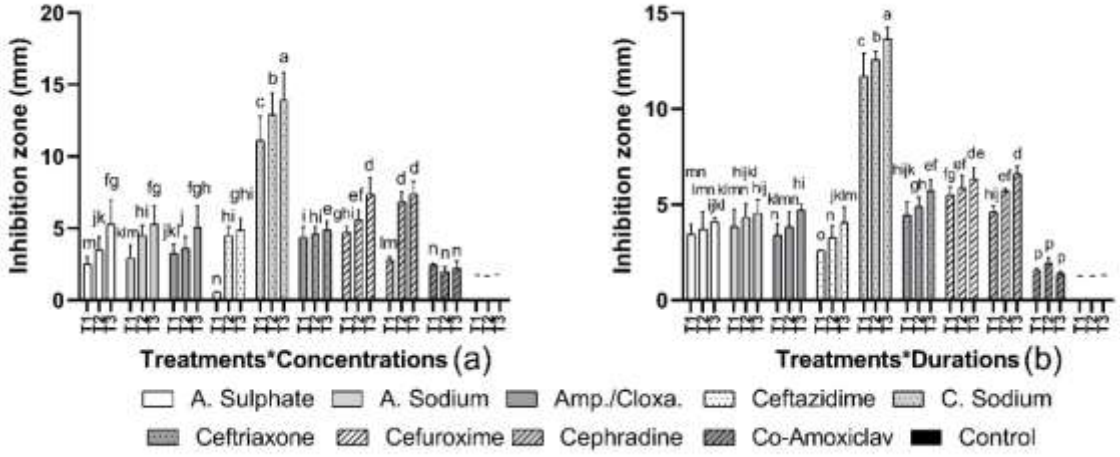

Figure 2: *In vitro* assessment of interaction between treatments with concentrations (a) and treatments with durations (b) against *X. axonopodis* pv. *eucalyptorum* through inhibition zone technique.

Analyzing the interaction between treatments and durations revealed that after 12 hours, 24 hours, and 36 hours, Ceftriaxone displayed the highest inhibition zone (12 mm, 13 mm, and 14 mm, respectively). This was followed by Cephradine, Ampicillin/Cloxacillin, Ampicillin Sodium, Cefuroxime, Ceftizoxime Sodium, Amikacin Sulphate, Co-amoxiclav, and Ceftazidime in their respective time durations, compared to the control treatment ((Figure 2b).

Assessment of antibiotics for the management of bacterial blight of *Eucalyptus* under greenhouse conditions

Among all four treatments, the combination of Ceftriaxone + Cephradine exhibited the lowest disease incidence of 15%, followed by Cephradine and Ceftriaxone, when compared to the control treatment under greenhouse conditions (Figure 3a). In terms of the interaction between treatments and concentrations, the combination of Ceftriaxone + Cephradine demonstrated the least disease incidence (19%, 16%, and 11%) at 300 ppm, 500 ppm, and 700 ppm concentrations, respectively. This was followed by Cephradine and Ceftriaxone at the same concentrations, all of which showed lower disease incidence compared to the control treatment (Figure 3b).

When considering the interaction between treatments and durations, the combination of Ceftriaxone + Cephradine displayed the minimum disease incidence (21%, 14%, and 10%) after 10, 20, and 30 days, respectively. This was followed by Cephradine and Ceftriaxone after the same durations, all of which exhibited lower disease incidence compared to the control treatment (Figure 3c).

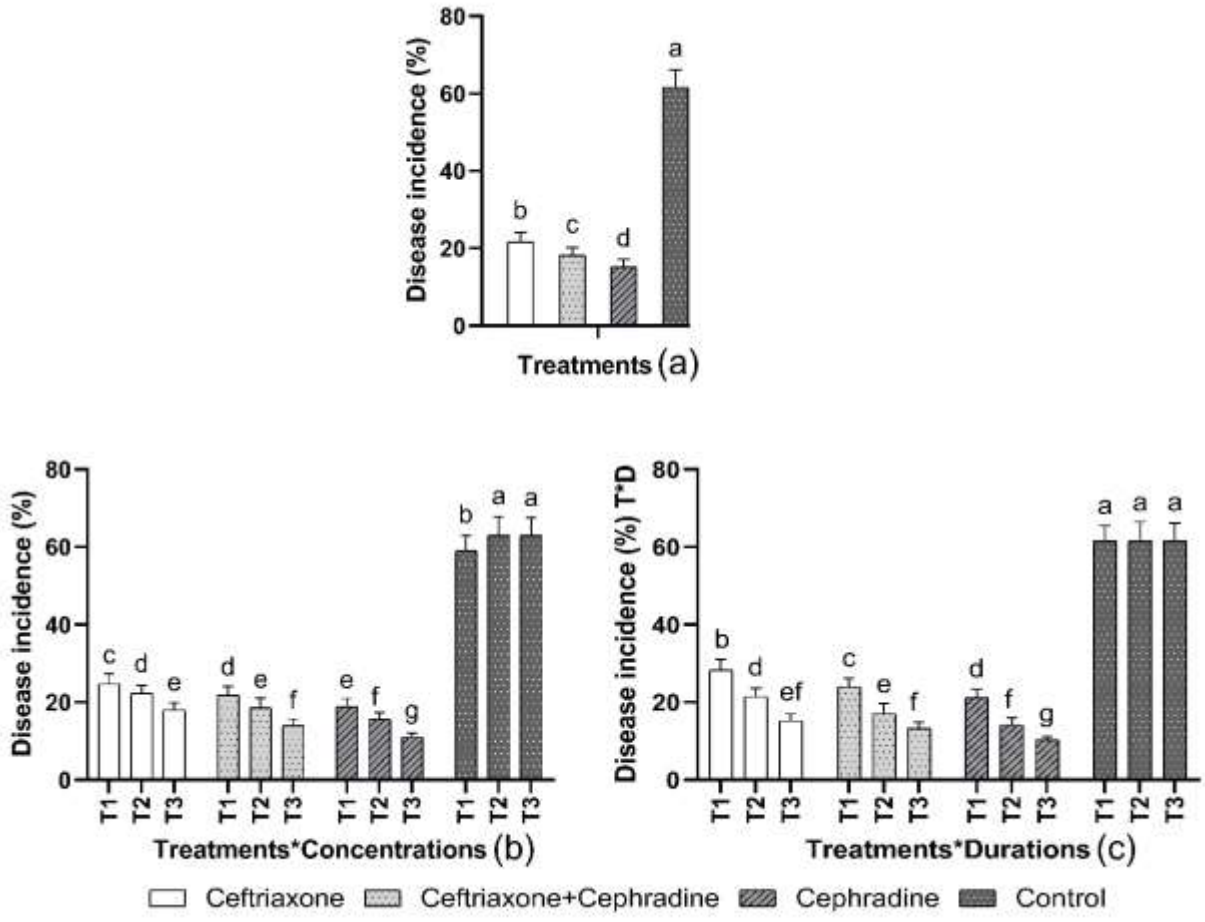

Figure 3: Assessment of antibiotics with different treatments (a), interaction between treatments with concentrations (b) and treatments with duration (c) against bacterial blight of Eucalyptus under greenhouse conditions.

***In vivo* assessment of antibiotics against Bacterial blight of Eucalyptus**

Among all the treatments, the combination of Ceftriaxone + Cephradine exhibited the lowest disease incidence of 18%, followed by Cephradine of 21% and Ceftriaxone of 25%, in comparison to the control treatment under field conditions ((Figure 4a).

Regarding the interaction between treatments and concentrations, the combination of Ceftriaxone + Cephradine demonstrated the least disease incidence (22%, 18%, and 14%) at 300 ppm, 500 ppm, and 700 ppm concentrations, respectively. This was followed by Cephradine and Ceftriaxone at the mentioned concentrations, all of which exhibited lower disease incidence compared to the control treatment (Figure 4b).

Considering the interaction between treatments and duration in terms of disease incidence, the combination of Ceftriaxone + Cephradine displayed the minimum disease incidence (24%, 18%, and 13%) after 10, 20, and 30 days, respectively. This was followed by Cephradine and Ceftriaxone after the same durations, both of which showed lower disease incidence compared to the control treatment (Figure 4c).

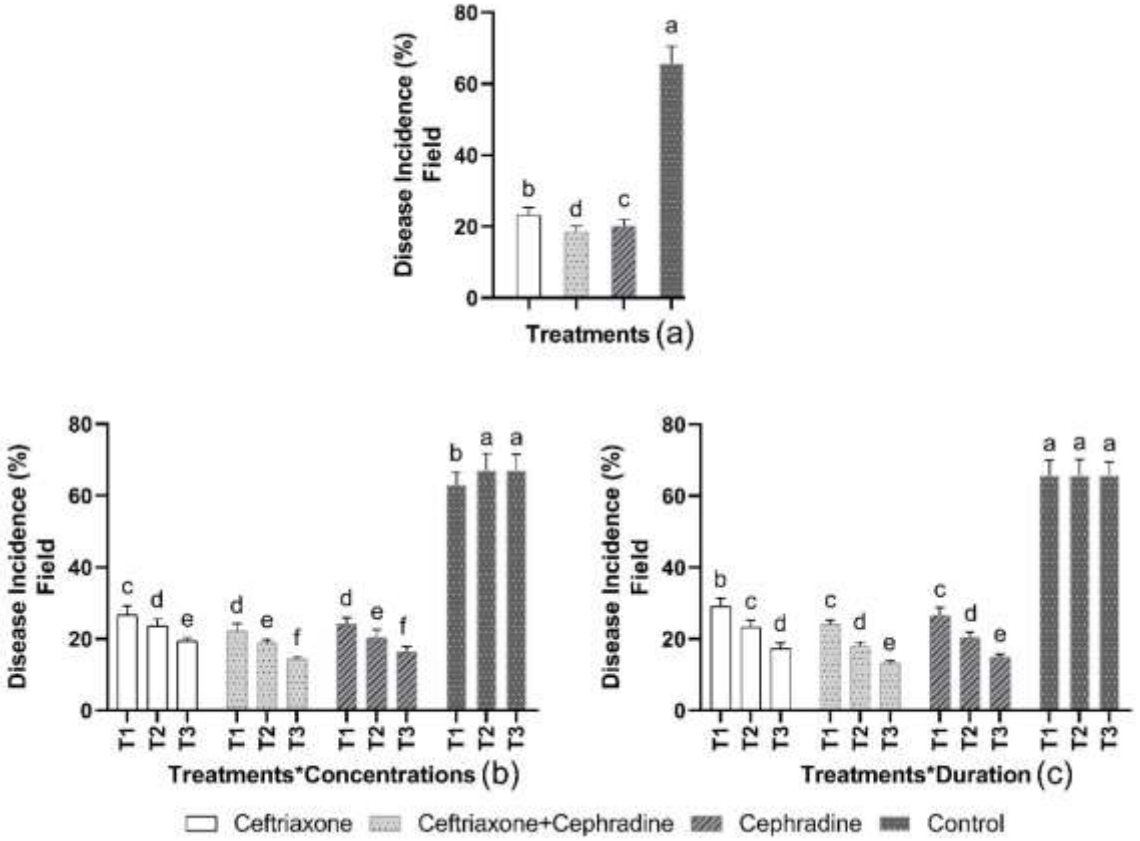

Figure 4: *In vivo* assessment with different treatments (a), interaction between treatments with concentrations (b) and treatments with duration (c) against bacterial blight of Eucalyptus under field conditions.

DISCUSSION

In the present study, various antibiotics were utilized to control bacterial leaf blight pathogens under laboratory, greenhouse, and field conditions. The utilization of antibiotics can effectively manage diseases in aquaculture and enhance production (Son et al., 1997; Li et al., 1999). However, the emergence and proliferation of drug-resistant strains pose significant challenges in developing uncomplicated and efficacious compounds (Gnanamanickam et al., 2014). Fitt et al. (1992) noted that specific antibiotics can regulate bacterial growth when introduced to culture

media. The study evaluated six antibiotics, namely Sino Bionic, Streptomycin Sulphate, Benzyl Penicillin Sodium, Kanamycin Sulphate, Chloramphenicol Sodium, and Ampicillin Sodium against *X. axonopodis*. Among these, Sino Bionic, Benzyl Penicillin Sodium, Streptomycin Sulphate, and Kanamycin Sulphate demonstrated effectiveness and exhibited significant zones of inhibition at concentrations of 31 µl, 62 µl, 125 µl, and 500 µl. The average inhibition zone sizes were 1.4 cm, 1.6 cm, 1.8 cm, and 2.2 cm, respectively, against *X. axonopodis* pv. *citri*. The concentration of 500 µl yielded the most favorable results, consistent with the findings of Mubeen et al. (2015).

The study employed three antibiotic concentrations viz. 300 ppm, 500 ppm, and 700 ppm for the inhibition zone technique. Notably, Ceftriaxone displayed the largest inhibition zone of 13 mm among all ten treatments. This outcome aligns with the work of Khatua et al. (2013), who also found Ceftriaxone effective against *Xanthomonas* bacteria in their assessment of antibiotic sensitivity. Similar evidence of Ceftriaxone's efficacy against *Xanthomonas* species is provided by Son et al. (1997).

Cephradine emerged as the second most effective antibiotic, demonstrating maximum inhibition of bacterial growth after Ceftriaxone. These results are consistent with the findings of Nadeem et al. (2016), who investigated the effects of various antibiotics on *Xanthomonas* isolates, including Cephradine. Mubeen et al. (2015) also reported the sensitivity of *Xanthomonas* to multiple antibiotics, including Streptomycin, Ceftriaxone, Cephradine, Kanamycin, and Streptocycline. Previous studies (Pruvost et al., 2005) have likewise documented significant inhibitory effects of Cephradine against bacterial growth.

Among the treatments evaluated in greenhouse and field conditions, the combination of Ceftriaxone + Cephradine exhibited the lowest disease incidence at 18%. These findings are consistent with the conclusions drawn by Khatua et al. (2013) and Parola et al. (2017), who observed the effectiveness of Ceftriaxone and Cephradine in field trials against bacterial blight in Eucalyptus. The efficacy of these antibiotics is also supported by the research of Ismail et al. (2016), who studied the impact of Cephradine and Ceftriaxone in field trials and assessed their effects by recording disease incidence.

CONCLUSION

The study provides valuable understandings for the management of bacterial blight disease in *Eucalyptus camaldulensis* using different antibiotics. The main conclusion of the study is that Ceftriaxone and the Cephradine + Ceftriaxone combination are the most effective treatments for reducing the disease incidence and severity under various conditions.

RECOMMENDATIONS

It is therefore, strongly recommended to maintain regular monitoring of nursery and field conditions, and to adjust antibiotic treatments accordingly in order to prevent or minimize disease outbreaks. Alongside this, the implementation of various other disease management strategies should be considered, including the utilization of planting materials that are resistant or tolerant to the disease, the adoption of effective sanitation measures, the application of suitable chemical treatments, and the introduction of biological control agents. These additional measures are intended to complement the antibiotic treatments and contribute to the overall improvement of the health and productivity of *Eucalyptus camaldulensis*.

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AUTHORS' CONTRIBUTIONS

MK conducted research experiments and wrote original manuscript; IA conceived and supervised research experiments; MA supervised lab experiments and provided technical assistance; MA provided technical assistance in field experiments; MHUR prepared graphs of the manuscript; SA critically reviewed the manuscript, HMFS checked the reference; WUD helped in manuscript write up; ZA helped in data collection.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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